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Optimizing a protein-specific ELISA for the detection of protein-marked insects

(Keywords: Protein-marking, biological control, mark–release–recapture, dispersal, *Hippodamia convergens*)

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Abstract. A series of tests were conducted to determine if the sensitivity and efficiency of an established rabbit immunoglobulin (IgG)-specific enzyme-linked immunosorbent assay (ELISA) could be improved for detecting a protein mark on insects. Five variations of ELISA were examined for their ability to detect a rabbit IgG mark on the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville. The conventional sandwich ELISA was the most sensitive immunoassay based on the strength of the ELISA reaction and the proportion of individuals scoring positive for rabbit IgG. ELISAs were also conducted on rabbit IgG-marked beetles that were either homogenized or soaked in sample buffer prior to the assay. Results showed that homogenized beetles yielded higher ELISA values in the conventional ELISA than soaked beetles, but the qualitative response (i.e., percentage scoring positive for the mark) was about the same for up to 18 days after marking. Therefore, in some instances, simply soaking an individual beetle might be a viable alternative to the labour-intensive homogenization of the sample. Sandwich ELISAs with immunoreagent incubation intervals held constant at 5, 10, 20, or 60 min were also examined for their ability to detect rabbit-IgG-marked beetles. Results showed that the ELISA with immunoreagent incubations of 60 min yielded a significantly higher ELISA reading than the shorter intervals. However, all the marked beetles examined scored positive for the presence of rabbit IgG, regardless of the incubation interval. Finally, a test was conducted to determine if the conventional ELISA could also detect the presence of relatively inexpensive normal rabbit serum on marked beetles. Beetles marked with rabbit serum diluted one part rabbit serum to one, four, or eight parts water yielded statistically similar ELISA values to those beetles marked with the conventional rabbit IgG mark.

1. Introduction

A protein-specific enzyme linked immunosorbent assay (ELISA) was developed over a decade ago for detecting the presence of purified vertebrate immunoglobulin G (IgG) blood serum protein marks on insects (Hagler *et al.*, 1992a). The ELISA was developed to provide researchers with an alternative mark–release–recapture (MRR) procedure that is, in some instances, more sensitive and/or less expensive than the conventional marking procedures (Southwood, 1966; Hagler and Jackson, 2001). This protein-detecting ELISA has been used successfully for identifying rabbit or chicken IgG marks on relatively large insect herbivores and predators and minute parasitoids (Hagler *et al.*, 1992a; Hagler, 1997a,b; Hagler and Jackson, 1998; Hagler and Miller, 2002). However, there is still potential to improve this protein-marking system for future MRR or mark–capture studies.

One way to improve the system may be to use a different ELISA format to analyse for the protein mark. The only ELISA format used thus far to detect protein marks on insects has been the sandwich ELISA (hereafter referred to as the conventional ELISA) (Hagler and Jackson, 2001). However, other ELISA formats, such as direct and indirect ELISA might be more precise, economical, and efficient (Crowther, 1995; Greenstone, 1996; Diamandis and Christopoulos, 1996; Hagler, 1998). Another way to improve the system would be to decrease the time and labour required to conduct the assay. Currently, it takes about 7 h from the start of sample preparation to the end of substrate incubation to conduct a conventional ELISA. However, no studies have addressed whether assay time can be reduced while maintaining assay sensitivity. If homogenization of individual insects in sample buffer, the most labour-intensive step of the conventional ELISA, might be avoided for externally marked insects the amount of labour could be significantly reduced. Finally, the cost of marking insects with protein could be significantly reduced if a less expensive protein was effective.

Here I report the results from several studies designed to improve the original protein-marking ELISA system (Hagler *et al.*, 1992a). First, adult lady beetles, *Hippodamia convergens* Guérin-Méneville were marked with rabbit IgG and assayed by five different ELISA configurations. Additionally, each assay was conducted on individual beetles that were either homogenized with a tissue grinder in sample buffer or soaked in sample buffer prior to analysis. Second, the sensitivity of the conventional sandwich ELISA for detecting rabbit IgG-marked beetles was compared to ELISAs with shorter incubation intervals. Finally, the conventional ELISA was tested for efficacy with rabbit IgG-marked beetles compared to beetles marked with less expensive normal rabbit serum.

2. Materials and methods

2.1. Detecting rabbit IgG-marked beetles by different ELISA formats

A test was conducted to determine the sensitivity of various ELISA formats for detecting rabbit IgG-marked beetles that were

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either homogenized or soaked in sample buffer prior to the assay.

2.1.1. Test insect. All assays were conducted on adult *H. convergens* (Coleoptera: Coccinellidae) of unknown age that were purchased from a supplier of beneficial insects (Nature's Control, Medford, OR, USA). The beetles were stored in a refrigerator at 4°C for up to 1 week until they were ready to be marked.

2.1.2. Marking procedure. Hundreds of beetles were removed from the refrigerator and placed into a 29 × 29 × 29 cm screen cage and held at 27°C. The beetles were marked with 5.0 ml of a 5.0 mg/ml solution of reagent grade rabbit IgG (Sigma Chemical Company, St. Louis, MO, USA, #I5006) using an airbrush (Paasche Airbrush Company, Harwood Heights, IL, USA). Briefly, 5.0 ml of a water solution containing 25 mg of rabbit IgG was placed into the airbrush reservoir. The airbrush was inserted into the cage and the beetles were sprayed until there was no more rabbit IgG solution remaining in the reservoir (≈2.0 min). The beetles were held in the container for 1 h after marking.

2.1.3. Sample preparation. Rabbit IgG-marked beetles were removed from the screen cage they were marked in and placed in a clean screen cage containing pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera) eggs for food and a wet sponge for water. Crumpled strips of paper placed in the cage provided a substrate for the beetles to walk on. The entire cage was held in an environmental chamber at 27°C:23°C (L:D), 40% relative humidity, and 14:10 h (L:D). Individual beetles ($n=30/\text{day}$) were removed from the cage 0, 2, 6, 10, 14, 18 and 22 days after marking and assayed by each ELISA described below. The beetles were either homogenized ($n=15/\text{day}$) or soaked ($n=15/\text{day}$) in 500 μl of tris buffered saline (TBS, pH 7.4).

2.1.4. Unmarked beetle controls. Beetles untreated with rabbit IgG (negative controls) were assayed by each of the ELISAs described below. A beetle marked with rabbit IgG was scored positive for IgG if its absorbance value exceeded the mean negative control reading by three standard deviations (Hagler, 1997a,b). Each ELISA plate contained 16 unmarked individuals.

2.1.5. ELISA procedures

2.1.5.1. Conventional ELISA. The conventional sandwich ELISA described by Hagler *et al.* (1992a) was performed on each beetle. Each of the 96 wells on an ELISA plate (Becton Dickinson Labware, Franklin Lakes, IL, USA, Falcon Pro-Bind, #353915) was coated with 100 μl of goat anti-rabbit IgG (1 mg/ml stock solution diluted 1:500 in dH_2O) (Sigma, #R2004) and incubated overnight at 4°C. Antibody was discarded and each well was then incubated with 350 μl of SuperBlock[®] (Pierce Biotechnology Inc., Rockford, IL, USA, #37535) for 30 min at 27°C to block non-specific antibody binding to the wells of the ELISA plate. The blocking solution was emptied from each plate and a 50 μl aliquot of homogenized or soaked beetle sample was placed in a well and incubated for 1 h at 37°C. Wells were washed three times with TBS-Tween 20 (0.05%) and twice with

TBS. A 50 μl aliquot of goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma, #A6154) diluted to 1:1000 in 1% non-fat dry milk (NFDM) was added to the wells of the plate. Plates were washed again as described above and 50 μl of horseradish peroxidase substrate solution (1-Step[™] ABTS, Pierce, #37615) was added to each well of the plate. After 2 h, the absorbance of each well was measured with a microplate reader (SpectraMAX 250, Molecular Devices, Sunnyvale, CA, USA) set at 405 nm.

2.1.5.2. Direct ELISA. A 50 μl aliquot of homogenized or soaked beetle sample was placed in a well of a 96-well assay plate. Each plate was incubated at 4°C overnight. Following incubation, the samples were discarded from each plate and a 350 μl aliquot of SuperBlock solution was added to each well for 30 min at 27°C. The blocking solution was emptied from each plate and a 50 μl aliquot of goat anti-rabbit IgG conjugated to horseradish peroxidase diluted to 1:1000 in 1% NFDM was added to the wells of the plate. Plates were washed again as described above and a 50 μl aliquot of substrate solution was added to each well of the plate. After 2 h, the absorbance of each well was measured with a microplate reader set at 405 nm.

2.1.5.3. Indirect ELISA. A 50 μl aliquot of a homogenized or soaked beetle sample was placed in a well of a 96-well assay plate. Each plate was incubated at 4°C overnight. Following incubation, the samples were discarded from each plate and a 350 μl aliquot of SuperBlock solution was added to each well for 30 min at 27°C. The blocking solution was emptied from each plate and a 100 μl aliquot of goat anti-rabbit IgG diluted to 1:500 in dH_2O was added to each well of the plate. Plates were washed again as described above and 50 μl of rabbit anti-goat IgG conjugated to horseradish peroxidase (Sigma, #A5420) diluted to 1:10 000 in 1% NFDM was added to the wells of the plate. Plates were washed again as described above and 50 μl of substrate solution was added to each well of the plate. After 2 h, the absorbance of each well was measured with a microplate reader set at 405 nm.

2.1.5.4. Biotinylated sandwich ELISA. A 100 μl aliquot of goat anti-rabbit IgG diluted 1:500 in TBS-Tween 20 was placed in each well of a 96-well ELISA plate and incubated overnight at 4°C. Following incubation, the antibody was discarded from each plate and a 350 μl aliquot of SuperBlock was added to each well for 30 min at 27°C. The blocking solution was emptied from each plate and a 50 μl aliquot of homogenized or soaked beetle sample was placed in each well of the plate. Each plate was incubated at 4°C overnight. Plates were washed as above and 50 μl of goat anti-rabbit IgG conjugated to biotin (Sigma, #B7389) diluted 1:5000 in TBS-Tween 20 was added to the wells of the plate for 1 h at 37°C. Plates were washed again and a 50 μl aliquot of a 1:10 000 solution of streptavidin (made from a stock solution containing 1.0 mg/ml of streptavidin conjugated to horseradish peroxidase mixed in 50% phosphate buffered saline and 50% glycerol) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA, #016-030-084) conjugated to horseradish peroxidase was added to each well for 1 h at 37°C. Plates were washed again and 50 μl of substrate solution was added to each well of the plate. After 2 h, the absorbance of each well was measured with a microplate reader set at 405 nm.

2.1.5.5. Biotinylated direct ELISA. A 50 μ l aliquot of homogenized or soaked beetle sample was placed in a well of a 96-well assay plate. Each plate was incubated at 4°C overnight. Following incubation, the samples were discarded from each plate and a 350 μ l aliquot of SuperBlock solution was added to each well for 30 min at 27°C. Plates were washed as above and a 50 μ l aliquot of goat anti-rabbit IgG conjugated to biotin diluted 1 : 5000 in TBS-Tween 20 was added to the wells of the plate for 1 h at 37°C. Plates were washed as described above and a 50 μ l aliquot of a 1 : 10 000 streptavidin solution conjugated to horseradish peroxidase was added to each well for 1 h at 37°C. Plates were washed again and 50 μ l of substrate solution was added to each well of the plate. After 2 h, the absorbance of each well was measured with a microplate reader set at 405 nm.

2.1.6. Data analysis. The mean \pm SD ELISA absorbance value and the percentage of beetles scoring positive for rabbit IgG by each ELISA format on each sample date after marking was tallied.

2.2. Detecting rabbit IgG-marked beetles by ELISAs with shortened incubation intervals

A test was conducted to determine if the incubation intervals of the conventional ELISA can be reduced without losing sensitivity for detecting rabbit IgG-marked beetles.

2.2.1. Marking procedure. The marking procedure described by Hagler (Hagler, 1997b) to mark small and delicate parasitoids was used to mark adult *H. convergens*. Beetles were removed from the screen cage described above and placed into a 2.5 l Rubbermaid® container. The container's lid had a 6 cm diameter hole covered with organic fabric to facilitate air exchange. The beetles were marked with 2.0 ml of a 5.0 mg/ml solution of reagent grade rabbit IgG using a medical nebulizer (Sunrise Medical, Somerset, PA, USA, Model #800D). A nebulizer, which is a common medical device used to deliver inhaled medications, produces a very fine, fog-like mist (Hagler, 1997b). Briefly, 2.0 ml of a water solution containing 10 mg of rabbit IgG was placed into the nebulizer. The hose of the nebulizer was inserted into a 2.5 cm hole (just slightly larger than the mouth of the nebulizer) that was punched out of the side of the Rubbermaid® container. The air outlet was turned on and the beetles were 'fogged' until there was no more rabbit IgG solution remaining in the nebulizer (\approx 2.0 min). The nebulizer was removed from the container and the 2.5 cm hole in the Rubbermaid® was plugged with a cork. The beetles were held in the container for 1 h after marking and then placed in a clean screen cage.

2.2.2. Sample preparation. Individual beetles ($n=240$ per treatment) were removed from the cage the day after marking and soaked for 1 h in 500 μ l of TBS. A 100 μ l aliquot from each beetle sample was assayed for the presence of rabbit IgG by the conventional ELISAs described below. Unmarked beetles were also assayed by each ELISA. Beetles marked with IgG were scored positive for the presence of rabbit IgG by the method described in section 2.1.4.

2.2.3. ELISA procedure. Four variations of the conventional ELISA described above were tested on IgG-marked beetles that

were soaked in sample buffer prior to analysis. The protocol for each ELISA was the same as above with three exceptions: (1) the incubation interval for the anti-rabbit IgG, blocking solution, insect sample, and anti-rabbit IgG conjugated to horseradish peroxidase were held constant at 5, 10, 20 or 60 min, (2) a faster reacting horseradish peroxidase substrate was used instead of the conventional 1-Step ABTS™ substrate solution (TMB Microwell 1 Component HRP Substrate, BioFX Laboratories, Owings Mills, MD, USA, #TMBW-0100-04), and (3) the absorbance of each well was measured for 15 min after the substrate was added with the microplate reader set at 650 nm.

2.2.4. Data analysis. The mean \pm SD ELISA absorbance values and the percentage of beetles scoring positive for IgG remains by each ELISA format was tallied for each treatment. The absorbance values yielded by the ELISAs with 5, 10, 20 and 60 min incubation intervals were analysed for statistical differences by the Kruskal-Wallis one-way ANOVA on ranks. A Tukey multiple comparison test was conducted to identify significant differences between the incubation interval treatments ($p < 0.05$) (SSPS Inc., Chicago, IL, USA, SigmaStat, Ver. 2.03, 1997).

2.3. Detecting rabbit IgG and rabbit serum-marked beetles by ELISA

A test was conducted to determine if relatively expensive purified rabbit IgG marker could be replaced with inexpensive whole rabbit serum without any loss in the efficacy of the conventional ELISA for detecting marked beetles.

2.3.1. Marking procedure. Beetles were removed from the screen cage described above and marked with 2.0 ml of a 5.0 mg/ml solution of reagent grade rabbit IgG using a medical nebulizer as described above (Hagler, 1997b) (see section 2.2.1). Similarly, beetles were marked using the nebulizer with 2.0 ml of pure normal rabbit serum (BioSource International, Camarillo, CA, USA, #PLN5001) or 2.0 ml of normal rabbit serum diluted 1 : 1, 1 : 4, 1 : 8, 1 : 16, 1 : 32, 1 : 64, 1 : 128, 1 : 256, and 1 : 512 in dH₂O.

2.3.2. Sample preparation. Individual beetles ($n=24$ to 80 per treatment) were removed from the cage the day after marking and soaked for 1 h in 500 μ l of TBS. A 100 μ l aliquot from each beetle sample was assayed for the presence of rabbit IgG or rabbit serum at the various dilutions given above by the conventional ELISA (see section 2.1.5.1). Unmarked beetles were also assayed by ELISA. Beetles marked with rabbit IgG or rabbit serum were scored positive for the presence of the mark by the method described in section 2.1.4.

2.3.3. Data analysis. The mean \pm SD ELISA absorbance values and percentage of beetles scoring positive for rabbit IgG or rabbit serum was tallied. ELISA absorbance values for beetles marked with rabbit IgG and the various concentrations of rabbit serum were analysed for statistical differences by a Kruskal-Wallis one way analysis of variance on ranks. A Dunn's multiple comparison test (the default multiple comparison test for studies with unequal sample sizes) was conducted to identify significant differences between the marking treatments ($p < 0.05$) (SigmaStat, Ver. 2.03).

3. Results and discussion

The conventional ELISA was used previously for MRR to detect rabbit and chicken IgG marks on *H. convergens* for a study of intercrop dispersal (Hagler and Naranjo, 2004), to detect rabbit IgG-marked *Eretmocerus* spp. for a study of dispersal of a parasitoid after an inundative release (Hagler *et al.*, 2002), and to detect rabbit IgG-marked *Apis mellifera* L. for a study of honey

bee interactions with nestmates within a colony (DeGrandi-Hoffman and Hagler, 2000). The rabbit IgG and chicken IgG-specific sandwich ELISAs described in these studies were similar. Here, I describe methods that might improve the protein-marking technique for future studies.

A wide variety of immunoassays have been developed over the past half century for studying various aspects of biological and medical research (Crook and Payne, 1980; Crowther, 1995;

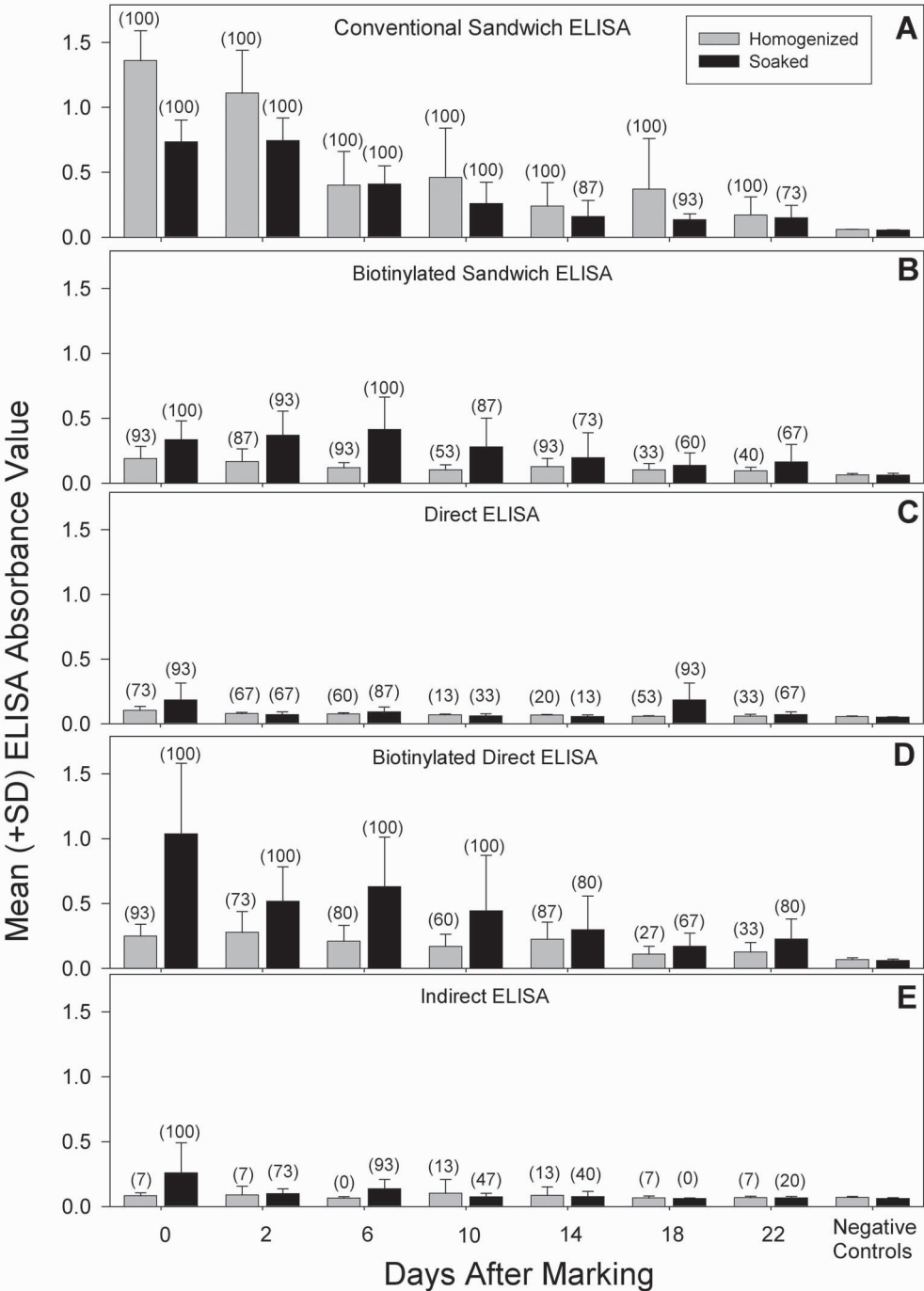


Figure 1. The mean (\pm SD) ELISA absorbance values and percentage (numbers above the error bars) of marked *Hippodamia convergens* scoring positive for the presence of rabbit IgG for up to 22 days after marking. Each individual was assayed by conventional ELISA (A), biotinylated sandwich ELISA (B), direct ELISA (C), biotinylated direct ELISA (D), and indirect ELISA (E). (n = 15 for each time interval and sample preparation [homogenized and soaked]).

Diamandis and Christopoulos, 1996; Greenstone, 1996). I have used pest-specific monoclonal antibodies in direct, indirect, and sandwich ELISAs and in dot blot and Western blot immunoassays to analyse predator gut contents for the presence of prey antigens (Hagler, 1998). The rabbit IgG-specific marking ELISA, a spin-off of the predator gut content ELISAs (Hagler *et al.*, 1991, 1992a,b), was developed to simultaneously monitor predator feeding activity and intercrop movement of inundative releases of biological control agents (Hagler *et al.*, 1992a; Hagler and Naranjo, 2004).

In the first test the conventional ELISA (Hagler *et al.*, 1992a; Hagler, 1997a,b; Hagler and Jackson, 1998; Hagler and Miller, 2002; Hagler and Naranjo, 2004) and four other ELISA formats were tested to determine their relative sensitivities at detecting rabbit IgG-marked *H. convergens*. There was considerable variation in both the quantitative (i.e., the ELISA absorbance values) and qualitative (i.e., the percentage of positive responses) outcome of the ELISAs. The conventional ELISA consistently yielded the highest ELISA readings and the fewest false negative immunoreactions throughout the duration of the study (figure 1A). These findings accord with a previous study in which rabbit IgG was retained well on beetles sprayed with marking solution for up to a month after release into a field cage (Hagler, 1997a). The indirect and direct ELISAs yielded the lowest ELISA absorbance values and the highest proportion of false negative immunoreactions, suggesting that they are unreliable for detecting rabbit IgG-marked beetles (figures 1C and 1E). The biotinylated ELISA formats were included in this study because they should, in theory, substantially amplify the targeted antigen (Bayer and Wilchek, 1996), however, the biotinylated ELISA formats proved to be less sensitive than the conventional ELISA format (figures 1B and 1D). Further tests using different concentrations of immunoreagents are in progress to determine if the sensitivity of the biotinylated ELISAs can be increased. Results from this study also showed that the probability of obtaining a negative immunoreaction generally

increased as the time since marking increased (figure 1). The decline in immunoreactivity and the subsequent higher incidence of false negative immunoreactions over time has been documented in previous studies on a wide variety of insect species (see Hagler references).

As mentioned above, the conventional ELISA takes about 7 h to complete from start to finish. However, about 90% of the time is comprised of labour-free incubation steps (i.e., adding the various immunoreagents and waiting). The most time-consuming and labour-intensive step of the conventional ELISA is the homogenization of individual insect samples in the sample buffer. As part of the first test, I compared the immunoreactivity of homogenized beetles with that of beetles soaked in sample buffer. With the exception of the conventional ELISA, the solution from soaked beetles generally yielded greater immunoreactions than the homogenized beetles (figure 1). While the soaked beetle solutions in the conventional ELISA yielded lower quantitative ELISA readings than the homogenized solutions, the percentage of individuals scoring positive by ELISA for rabbit IgG was at or almost at 100% for 18 days after marking. These data indicate that the tedious and time consuming process of homogenizing lady beetles may not be required for MRR studies lasting up to 18 days.

In the second marking test, the incubation intervals for each step of the sandwich ELISA were held constant for 5, 10, 20 or 60 min. Each interval yielded significantly different absorbance values with the ELISA readings increasing as the incubation intervals increased (figure 2). Even though the shorter incubation intervals yielded lower absorbance values, the qualitative efficacy of each ELISA interval remained 100%.

In the third marking test, beetles were marked with either rabbit IgG or various dilutions of normal rabbit serum. Rabbit IgG is a component of normal rabbit serum which has been isolated from the whole serum by fractionation and ion-exchange chromatography. Normal rabbit serum is whole serum obtained from non-immunized rabbits. The highest absorbance value was yielded by the rabbit IgG-marked beetles (figure 3). However, beetles marked with some of the more concentrated rabbit serum dilutions also yielded significantly high ELISA absorbance values (figure 3). Moreover, virtually every beetle marked with the various normal rabbit serum dilutions yielded a positive ELISA response. The beetles marked with pure rabbit serum yielded lower absorbance values than beetles marked with some of the less diluted rabbit serum concentrations tested. This discrepancy might be attributed to day-to-day variability of the ELISA protocol (Fenlon and Sopp, 1991) or the protein marking protocol (personal observation). This test was conducted to try to identify a less expensive protein mark than rabbit IgG. The highly purified reagent grade rabbit and chicken IgGs used thus far to mark insects for MRR studies cost US \$2.90 and US \$6.80 per mg, respectively. Therefore the cost of marking the beetles used in this study was US \$29.00. Conversely, normal rabbit serum only costs US \$5.00 per ml. If a 2.0 ml solution is diluted to one part serum to eight parts water (the most dilute concentration of rabbit serum that was not statistically different from rabbit IgG) the cost is US \$1.25. While the difference in cost was not significant in this smaller experiment, there are instances where the use of rabbit serum would lead to a significant reduction in the cost of marking insects. For example, in a recent study I showed that 80 mg (2.0 ml of IgG at 40 mg/ml) of rabbit IgG was much more

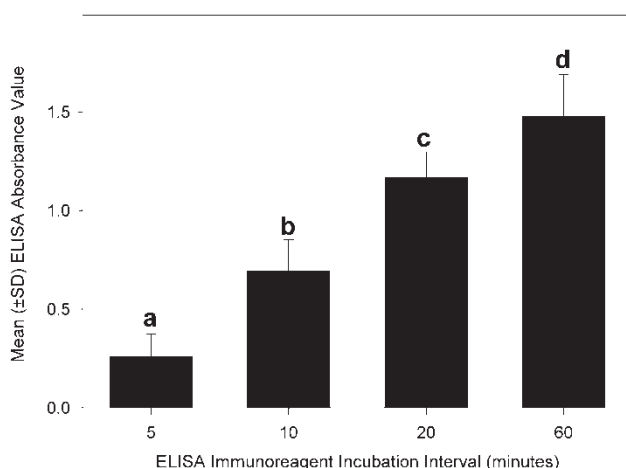


Figure 2. The mean (\pm SD) ELISA absorbance values of marked *Hippodamia convergens* assayed by ELISAs with immunoreagent incubation intervals held constant at 5, 10, 20 or 60 min. The letters above each bar indicates significantly different ELISA readings as determined by the Tukey's multiple comparison test ($p < 0.05$, $n=240$ per incubation interval). Every treated beetle, regardless of the ELISA incubation interval scored positive for the presence of rabbit IgG.

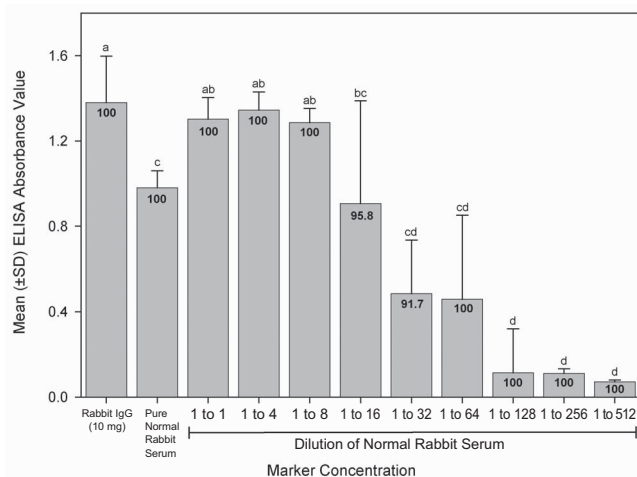


Figure 3. The mean (\pm SD) ELISA absorbance values and percentage (numbers in each bar) of IgG-marked *Hippodamia convergens* scoring positive for the presence of rabbit IgG or normal rabbit serum (at various dilutions). Significant differences in ELISA absorbance values for each treatment were determined by a Dunn's multiple comparison test ($p < 0.05$, $n = 80$ for the rabbit IgG and pure normal rabbit serum treatments and 24 for the various dilutions of normal rabbit serum).

efficient than 10, 20, or 40 mg for marking *Eretmocerus* spp. (Hagler *et al.*, 2002). Currently we are testing the efficacy of marking *Eretmocerus* spp. using diluted rabbit serum (in Prep.). Using a 1:8 dilution of rabbit serum instead of 80 mg of rabbit IgG would result in a 186-fold decrease in the cost of marking parasitoids.

In summary, this study shows that there is great variation in the ability of the different ELISAs to detect rabbit IgG-marks in predatory beetles. The conventional protein-marking ELISA described over a decade ago remains superior to the more recent ELISA protocols tested. The labour required to conduct the ELISA can be reduced by approximately 50% while maintaining efficacy by soaking the insect instead of homogenizing it in sample buffer. Furthermore, I recommend incubation intervals of 60 min for each step of the ELISA due to the decreased sensitivity observed for ELISAs with shorter incubation intervals. However, the blocking step can be reduced to 30 min. Finally, diluted rabbit serum may be a suitable marker alternative to rabbit IgG for many MRR studies. I am currently investigating the potential of using even less expensive markers for MRR and mark-capture studies.

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References

- BAYER, E. A. and WILCHEK, M., 1996. The avidin-biotin system. In E. P. Diamandis and T. K. Christopoulos (eds) *Immunoassay* (San Diego, CA: Academic Press), pp. 237–267.
- CROOK, N. E. and PAYNE, C. C., 1980. Comparison of three methods of ELISA for baculoviruses. *Journal of General Virology*, **46**, 29–37.
- CROWTHER, J. R., 1995. ELISA: Theory and Practice. In J. R. Crowther (ed.) *Methods in Molecular Biology Vol. 42* (Totowa, NJ: Humana Press Inc.), p. 223.
- DEGRANDI-HOFFMAN, G. and HAGLER, J., 2000. The flow of incoming nectar through a honey bee (*Apis mellifera* L.) colony as revealed by a protein marker. *Insectes Sociaux*, **47**, 302–306.
- DIAMANDIS, E. P. and CHRISTOPOULOS, T. K., 1996. In E. P. Diamandis and T. K. Christopoulos (eds) *Immunoassay* (San Diego, CA: Academic Press), p. 579.
- FENLON, J. S. and SOPP, P. I., 1991. Some statistical considerations in the determination of thresholds in ELISA. *Annals of Applied Biology*, **119**, 177–189.
- GREENSTONE, M. H., 1996. Serological analysis of arthropod predation: Past, present and future. In W. O. C. Symondson and E. Liddell (eds) *The Ecology of Agricultural Pests: Biochemical Approaches* (London, UK: Chapman & Hall), pp. 265–300.
- HAGLER, J. R., 1997a. Field retention of a novel mark-release-recapture method. *Environmental Entomology*, **26**, 1079–1086.
- HAGLER, J. R., 1997b. Protein marking insects for mark-release-recapture studies. *Trends in Entomology*, **1**, 105–115.
- HAGLER, J. R., 1998. Variation in the efficacy of several predator gut content immunoassays. *Biological Control*, **12**, 25–32.
- HAGLER, J. R., COHEN, A. C., ENRIQUEZ, F. J. and BRADLEY-DUNLOP, D., 1991. An egg specific monoclonal antibody to *Lygus hesperus*. *Biological Control*, **1**, 75–80.
- HAGLER, J. R., COHEN, A. C., BRADLEY-DUNLOP, D. and ENRIQUEZ, F. J., 1992a. A new approach to mark insects for feeding and dispersal studies. *Environmental Entomology*, **21**, 20–25.
- HAGLER, J. R., COHEN, A. C., BRADLEY-DUNLOP, D. and ENRIQUEZ, F. J., 1992b. Field evaluation of predation on *Lygus hesperus* using a species- and stage-specific monoclonal antibody. *Environmental Entomology*, **21**, 896–900.
- HAGLER, J. R. and JACKSON, C. G., 1998. An immunomarking technique for labeling minute parasitoids. *Environmental Entomology*, **27**, 1010–1016.
- HAGLER, J. R. and JACKSON, C. G., 2001. Methods for marking insects: current techniques and future prospects. *Annual Review of Entomology*, **46**, 511–543.
- HAGLER, J. R., JACKSON, C. G., HENNEBERRY, T. J. and GOULD, J. R., 2002. Parasitoid mark-release-recapture techniques – II. Development and application of a protein marking technique for *Eretmocerus* spp., parasitoids of *Bemisia tabaci*. *Biocontrol Science and Technology*, **12**, 661–675.
- HAGLER, J. R. and MILLER, E., 2002. An alternative to conventional insect marking procedures: detection of a protein mark on pink bollworm by ELISA. *Entomologia Experimentalis et Applicata*, **103**, 1–9.
- HAGLER, J. R. and NARANJO, S. E., 2004. A multiple ELISA system for simultaneously monitoring intercrop movement and feeding activity of mass-released predators. *International Journal of Pest Management*, **50**(3), 199–207.
- SOUTHWOOD, T. R. E., 1966. Absolute population estimates using marking techniques. In T. R. E. Southwood (ed) *Ecological Methods* (UK: Chapman & Hall), pp. 57–98.